Supplementary Material

RAN translation in Huntington disease

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Supplementary Figure 1. RAN translation across HTTexon1 and validation of HD-RAN C-terminal antibodies.

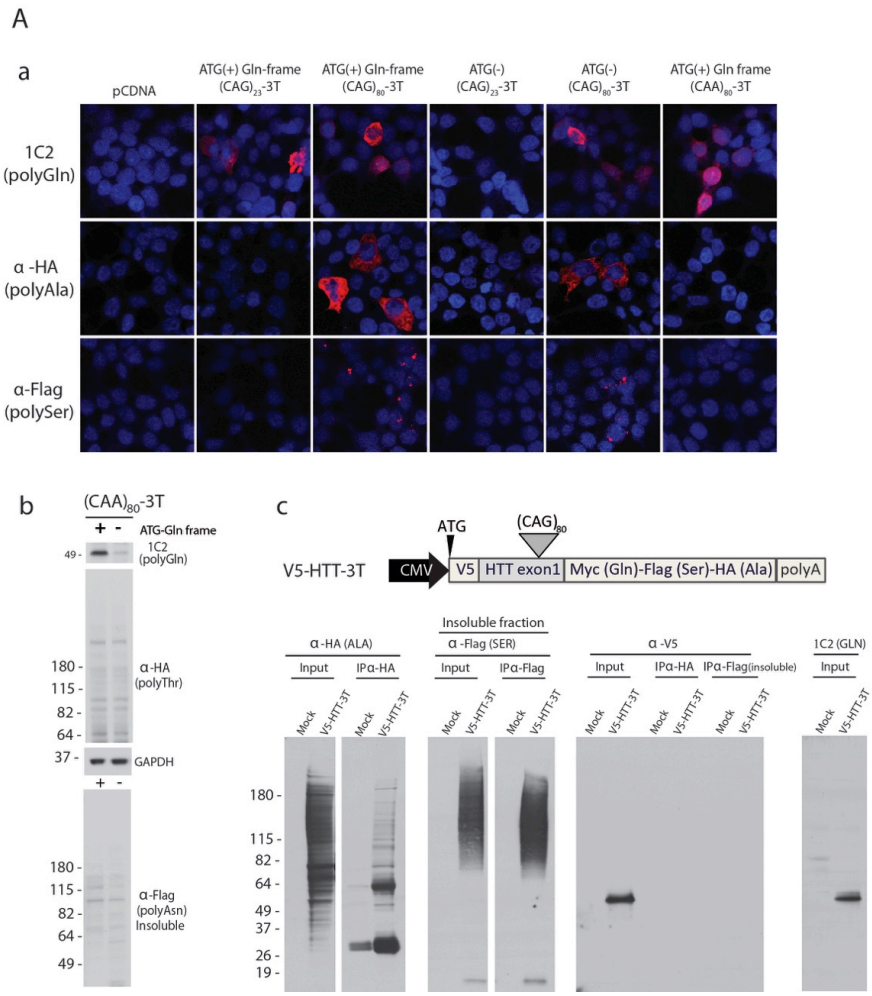


Figure S1A. RAN translation in cells transfected with HTTexon1 minigenes. (a) IF showing (+)ATG and RAN proteins expressed in cells transfected with 23 or 80 CAG or 80 CAA repeats +/- ATG (Gln frame). PolyGln is expressed from all constructs with an ATG in the polyGln reading frame (23 & 80 CAGs and 80 CAAs). RAN-polyGln is also expressed with 80 CAG repeats from ATG(-) minigene. RAN PolyAla shows diffuse cytoplasmic staining, RAN polySer forms multiple cytoplasmic aggregates and ATG/RAN polyGln shows nuclear and cytoplasmic staining. The minigene with 80 CAA repeats expressed ATG-polyGln but not RAN proteins in the other two reading frames. Red=positive staining, blue=DAPI. (b) Protein blots of HEK293T cells transfected with non-hairpin forming CAA constructs +/- ATG (Gln frame). Protein expression is detected in the polyGln reading frame with the +ATG CAA construct but not in alternative CAA reading frames (Asn or Thr). (c) Immunoprecipitation of lysates from HEK293T cells transfected with HTTexon1 minigenes with a 5'V5 N-terminal tag in the glutamine frame and 3 C-terminal tags in each reading frame. IPs using antibodies against C-terminal epitope tags in the polyAla and polySer frames are not positive for the N-terminal V5-Tag in the polyGln frame. These data demonstrate that frameshifing from the polyGln to the polyAla and polySer frames does not substantially contribute to the robust polyAla and polySer.

B

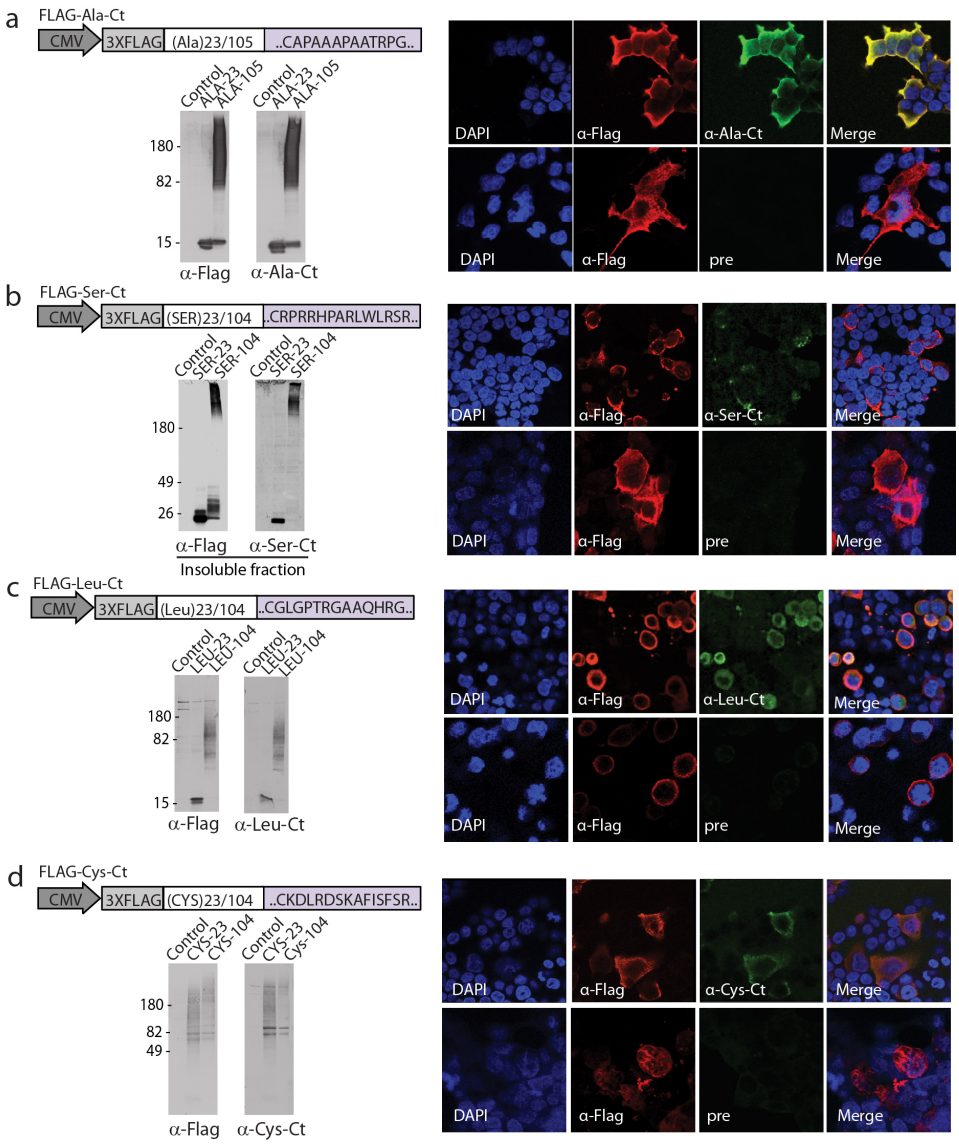


Figure S1 B. Validation of novel antibodies against C-terminal regions of putative HD-RAN polyAla, polySer, polyLeu and polyCys proteins. (a-d) Constructs used to express flag-tagged proteins with endogenous C-terminal regions, with sequences of peptides used to generate C-terminal antibodies indicated. HEK293T lysates assayed by protein blot (left) show detection of recombinant proteins by α-Flag and HD-RAN antibodies. The polyAla and polyLeu expansion proteins run as high-MW smears and the polySer expansion runs at the top of the gel. The more intense high MW smear of the short Cys-23 compared to the longer Cys-104 in panel S1Cd is likely caused by increased aggregation and insolubility of the long polyCys protein which does not enter the gel. These aberrant migration patterns suggest that these homopolymeric expansion proteins are insoluble and aggregation prone. It is also possible that these proteins can undergo posttranslational modifications. IF was performed on cells transfected with Flag-Ala(104 repeats), Flag-Ser (23 repeats), Flag-Leu (104 repeats) and Flag-Cys(23 repeats). IF shows co-localization of α-Flag (red) and newly developed HD-RAN C-terminal antibodies, α-polyAla-Ct, α-polySer-Ct, α-polyLeu-Ct and α-polyCys-Ct, (green).

Supplementary Figure2. HD-RAN, polyGln, Iba1 and Caspase3 staining in striatal brain regions.

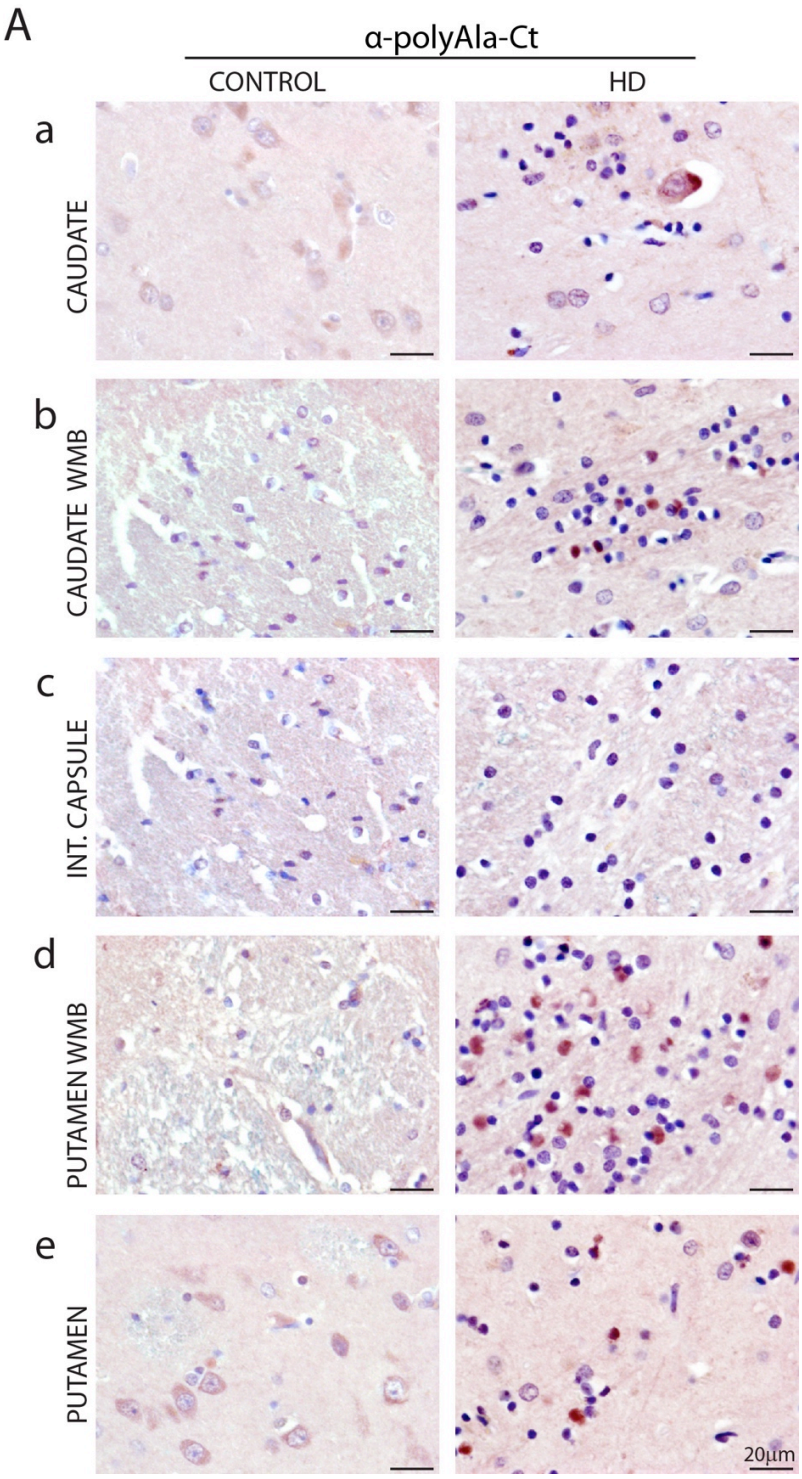


Figure S2 A. RAN-polyAla staining in striatal brain regions. IHC shows α-polyAla positive cells in human HD caudate (a) and putamen (e). α-polyAla staining is particularly abundant in the white matter bundles of both caudate and putamen (b,d). Internal capsule (c) is negative for α-polyAla staining. Red=positive staining, blue=nuclear counterstain.

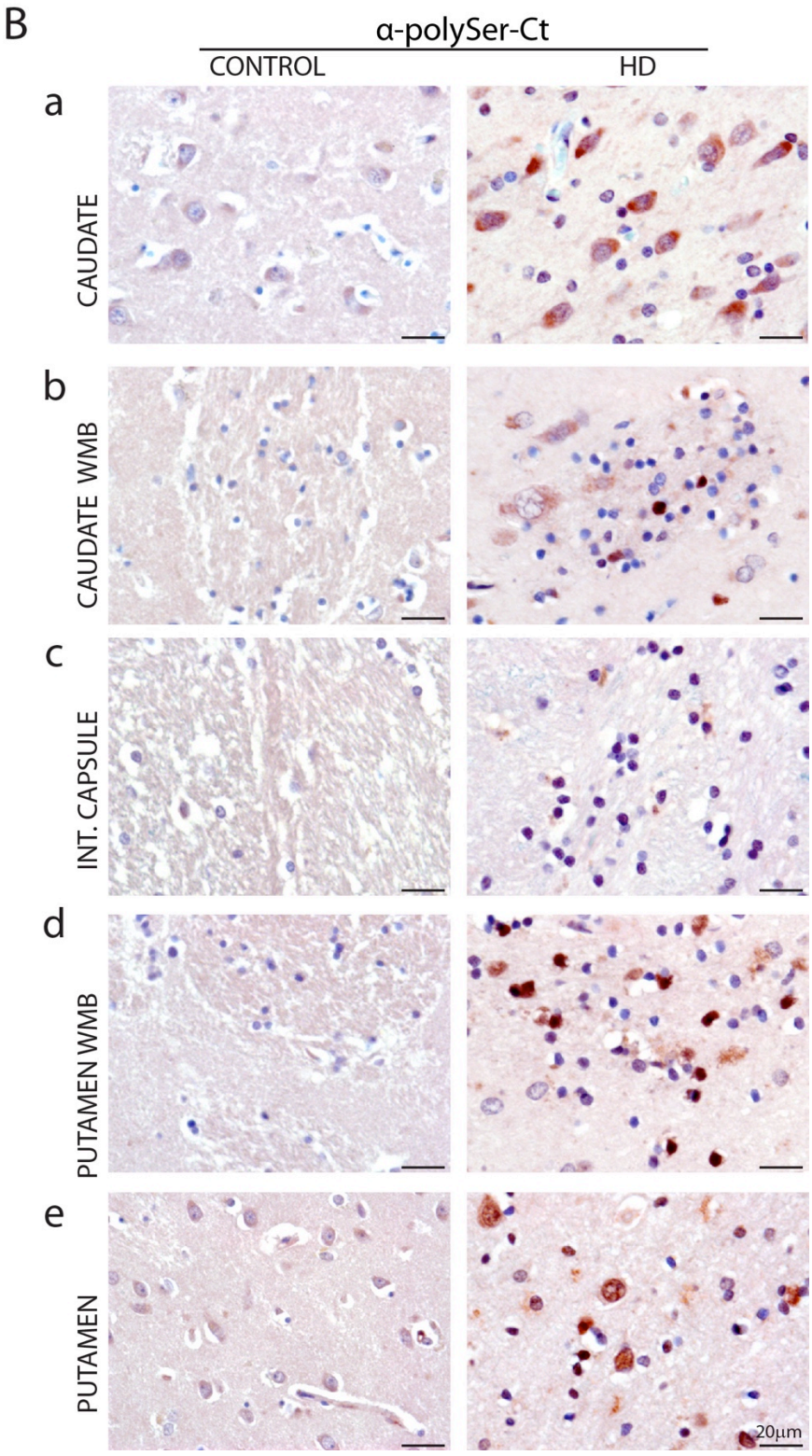


Figure S2 B. RAN-polySer staining in striatal brain regions. IHC shows α-polySer staining in caudate and putamen of the striatum and in the white matter bundles (a,b,d,e) of HD but not control autopsy samples. Staining is not found in the internal capsule (c). Poly-Ser localizes in both nucleus and cytoplasm. Red=positive staining, blue=nuclear counterstain.

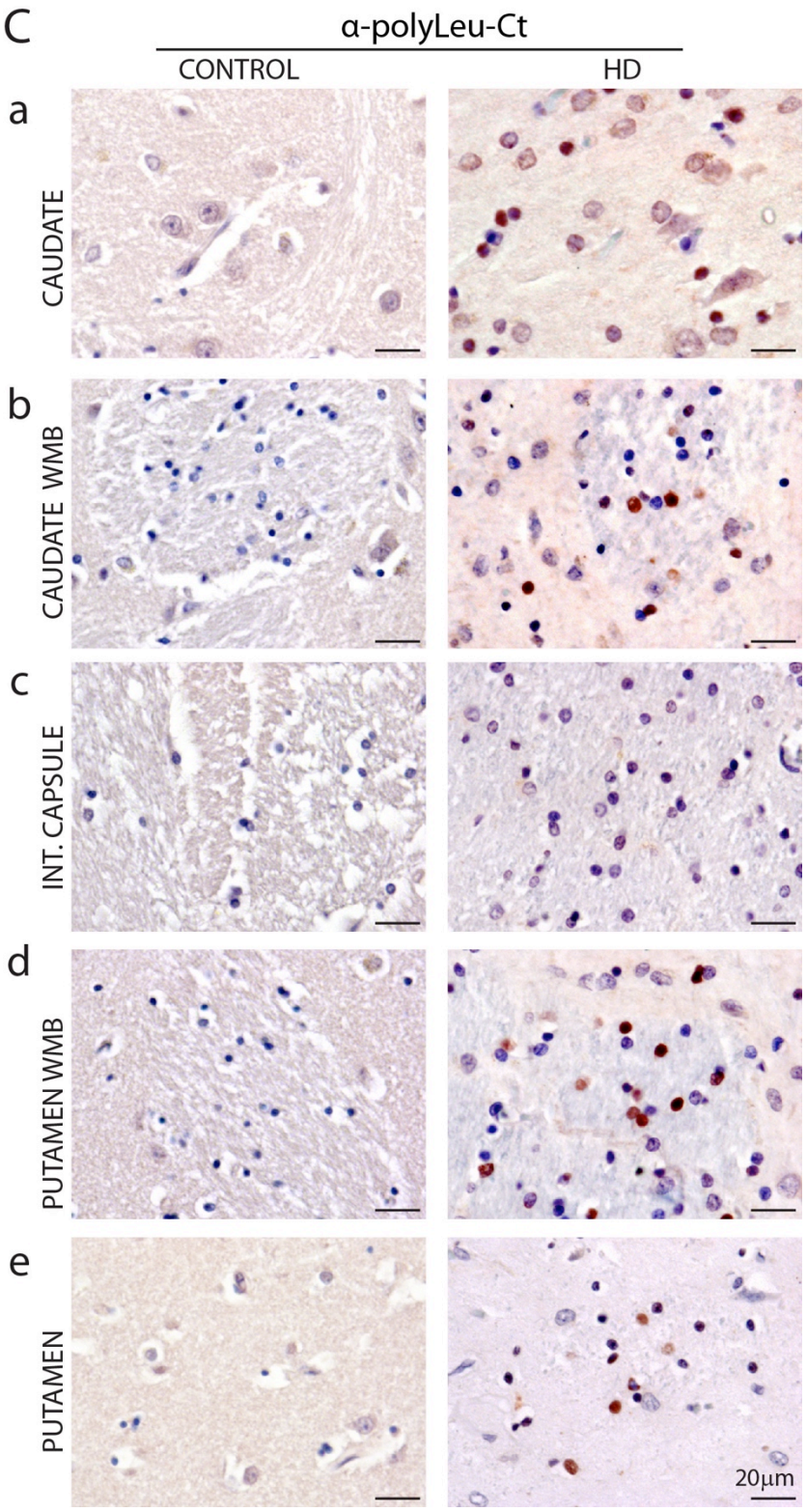


Figure S2 C. RAN-PolyLeu nuclear staining in HD striatum. IHC shows α-polyLeu nuclear staining in caudate, putamen and white matter bundles in human HD but not control autopsy tissue (a,b,d,e). In contrast, similar staining was not found in the internal capsule (c). Red=positive staining, blue=nuclear counterstain.

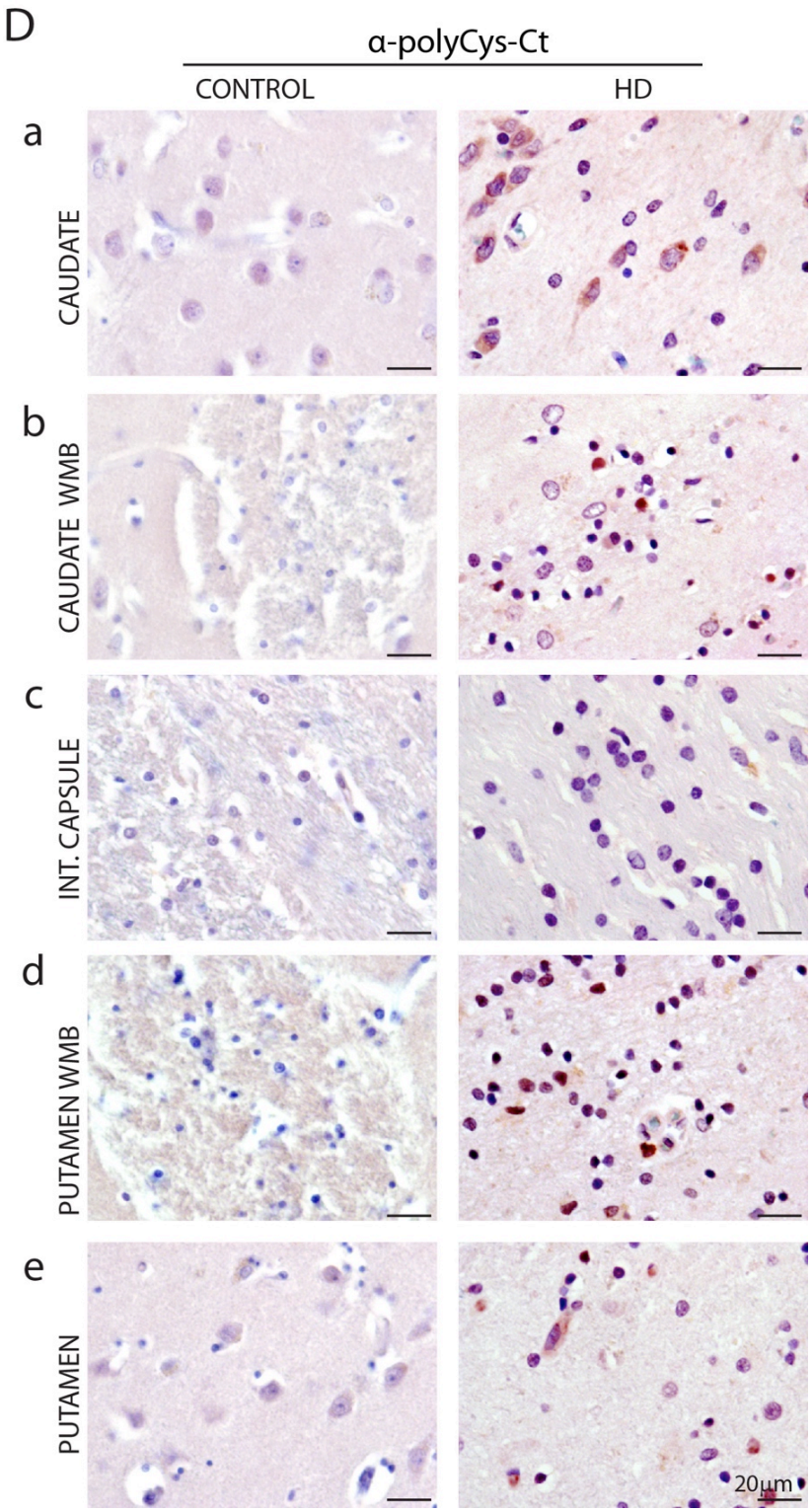


Figure S2 D. RAN PolyCys staining in HD striatum. IHC shows α-polyCys staining in caudate, putamen and white matter bundles in human HD but not control autopsy tissue (a,b,d,e). Similar staining was not found in the internal capsule (c). α-polyCys staining was nuclear or cytoplasmic with intense punctate staining seen in some cells. Red=positive staining, blue=nuclear counterstain.

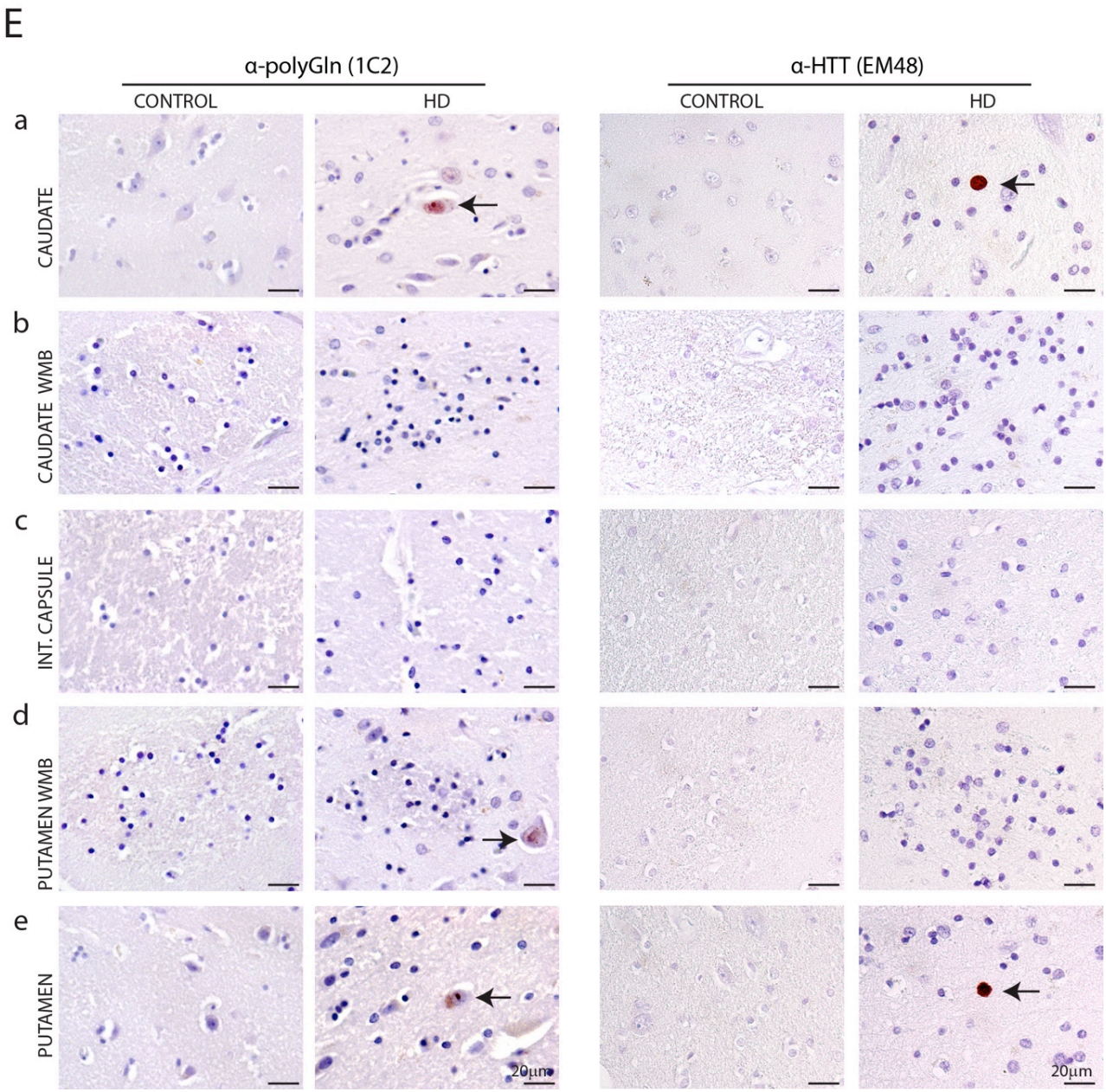


Figure S2 E. 1C2 staining shows nuclear and cytoplasmic polyGln aggregates in HD but not control caudate and putamen (a,e). Staining was negative in white matter bundles of the caudate and putamen (b,d) and internal capsule (c) at the experimental conditions tested. Comparable results were obtained using the EM48 antibody to detect HTT polyGln aggregates. Red=positive staining, blue=nuclear counterstain. Arrows highlight positive staining. 1C2 positive cell in panel D is located outside the circular white matter bundle region. Red=positive staining, blue=nuclear counterstain.

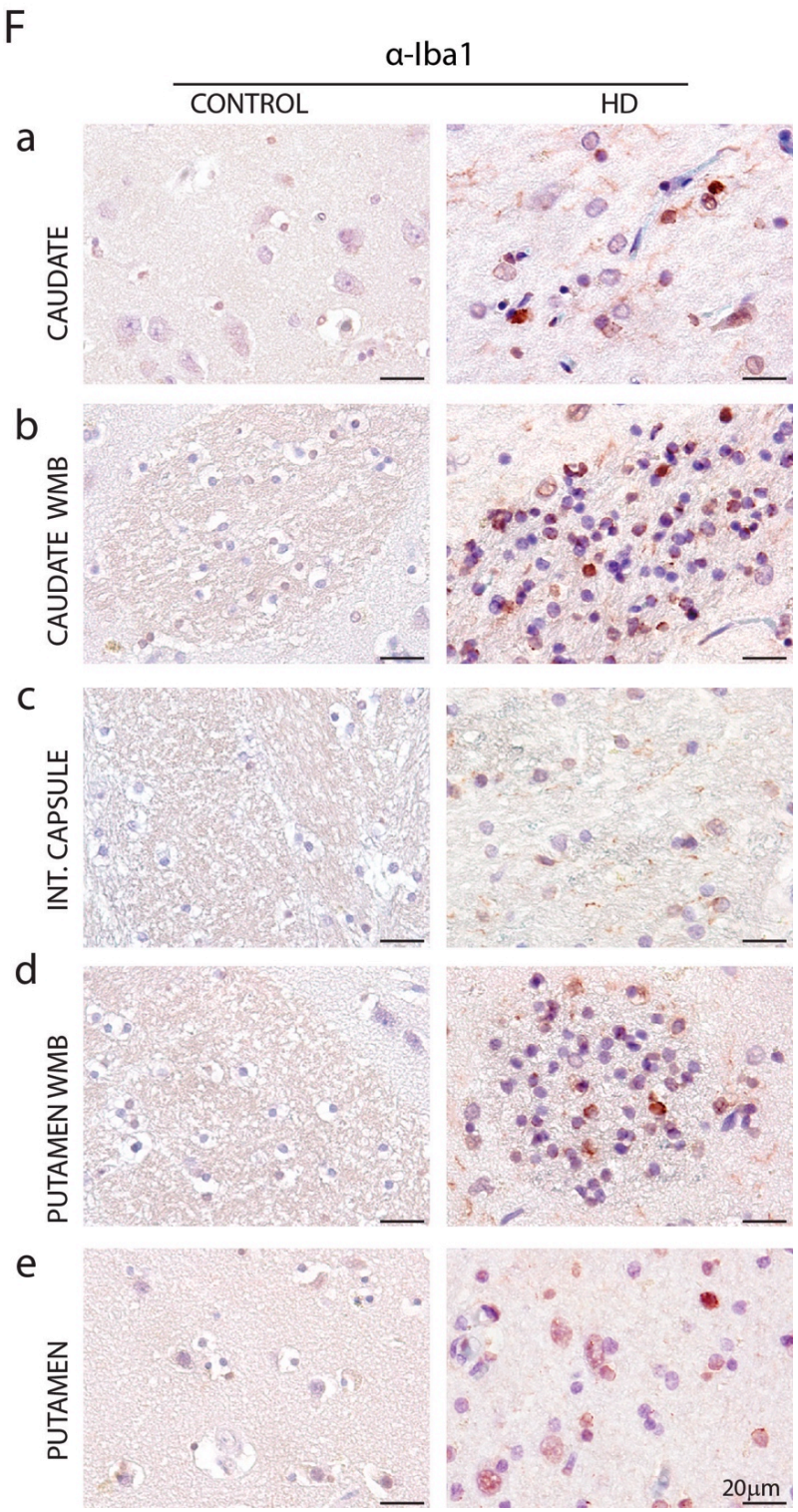


Figure S2 F. Iba1-positive microglia in HD striatum. IHC immunostaining for microglia with α-Iba1 shows strong immunostaining, similar to HD-RAN antibody staining, with positive signal in HD but not control caudate, putamen and white-matter bundles (a,b,d,e). Staining was not detected in the internal capsule (c). Red=positive staining, blue=nuclear counterstain.

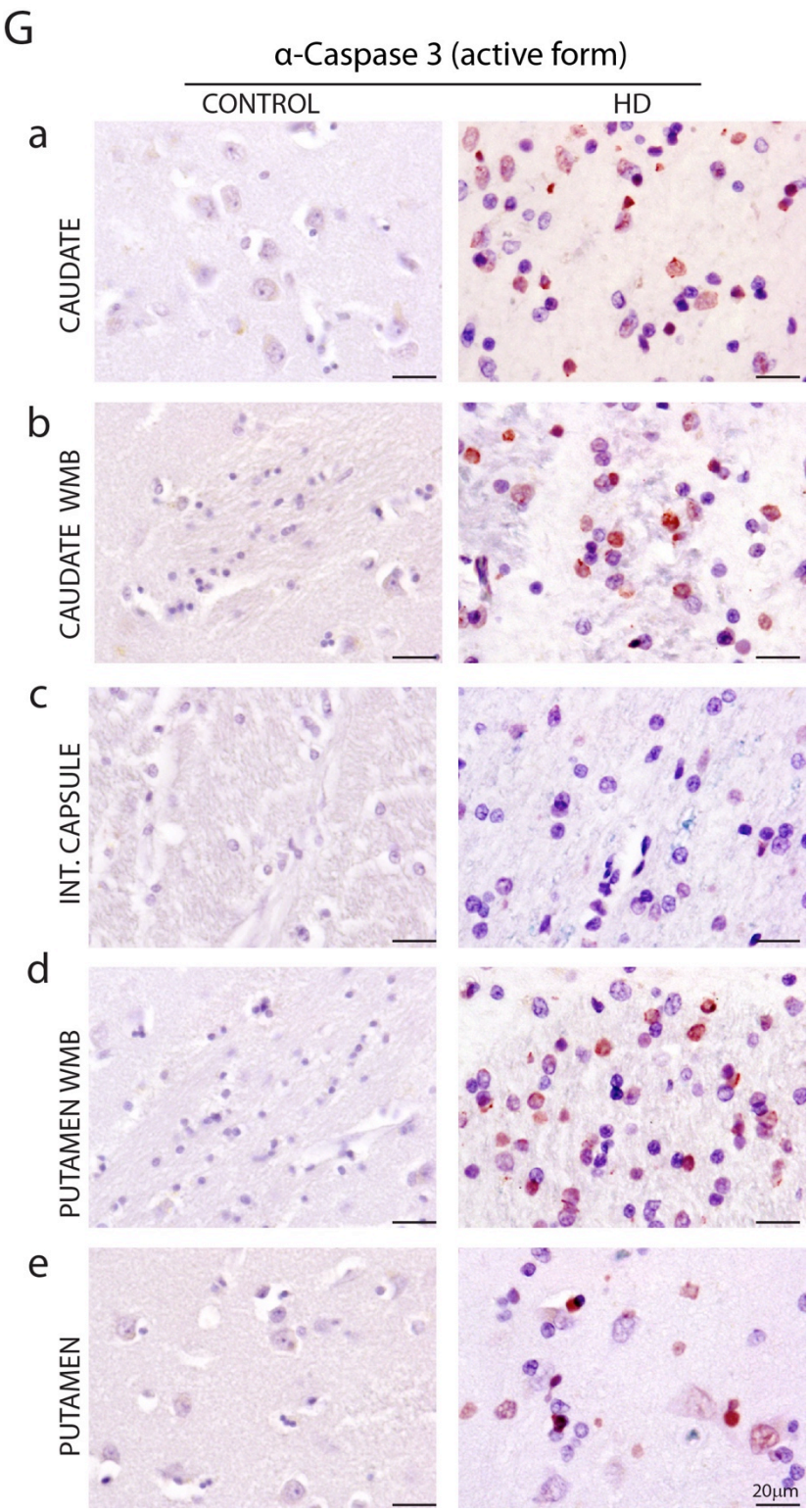


Figure S2 G. Active Caspase 3 staining in RAN-positive regions of the HD striatum (red). IHC shows frequent active caspase3 signal in caudate, putamen, and white matter bundles (a,b,d,e) of HD but not control striatum. Internal capsule also shows α-caspase 3 positive cells, although rare. Red=positive blue=nuclear counterstain.

Supplementary Figure 3. HD-RAN and polyGln staining in frontal cortex and cerebellum.

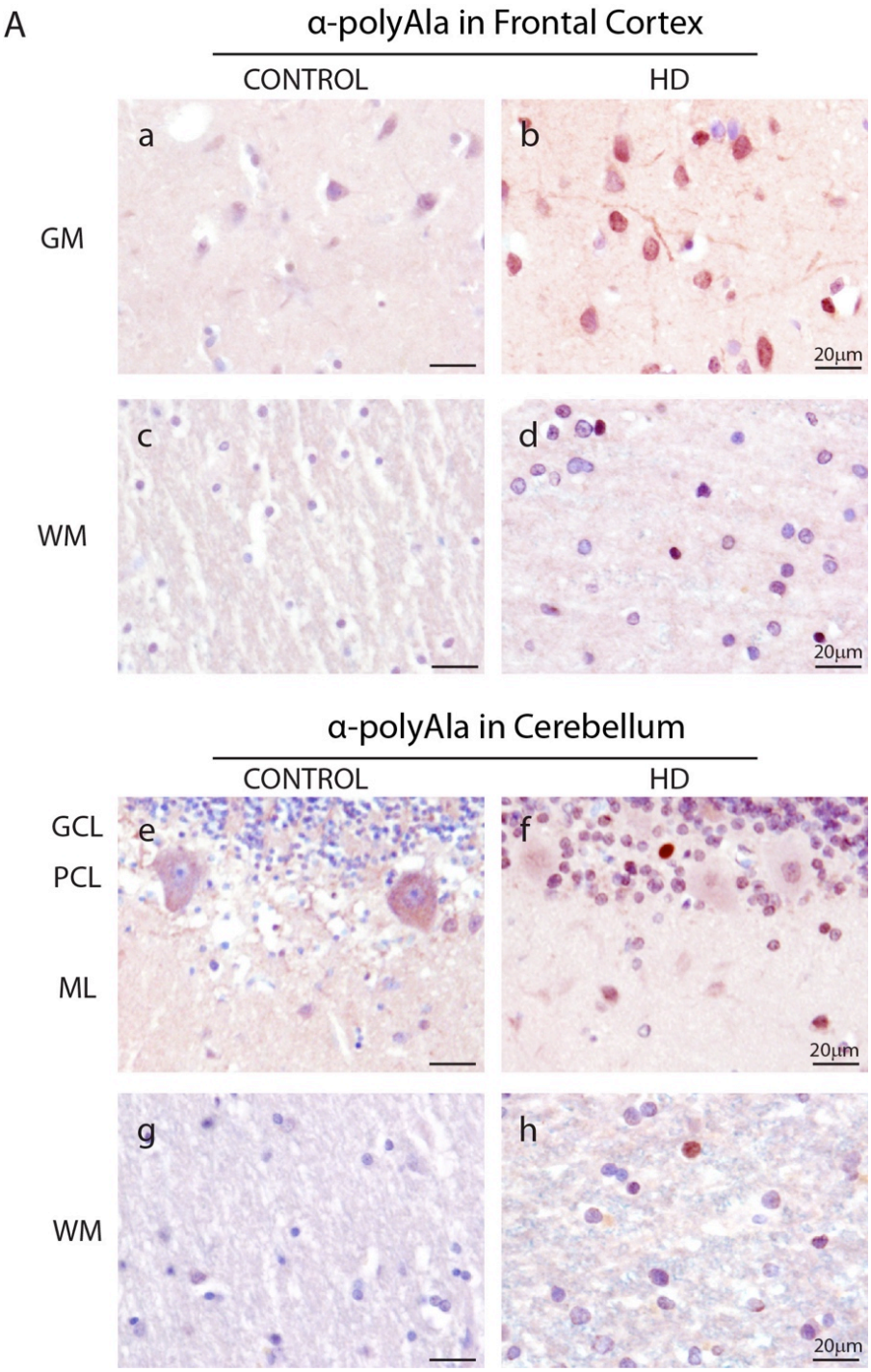


Figure S3A. PolyAla RAN proteins in HD frontal cortex and cerebellum. IHC staining shows positive α-polyAla staining in grey and white matter of HD (b,d) but not control (a,c) frontal cortex. Similarly, α-polyAla staining was found in cerebellar granular cell layer, molecular layer and deep white-matter regions of the cerebellum in HD (f,h) but not control samples (e,g). GM=grey matter, WM=white matter, GCL=granule-cell layer, PCL= Purkinje-cell layer, ML=molecular layer. Red=positive staining, blue=nuclear counterstain.

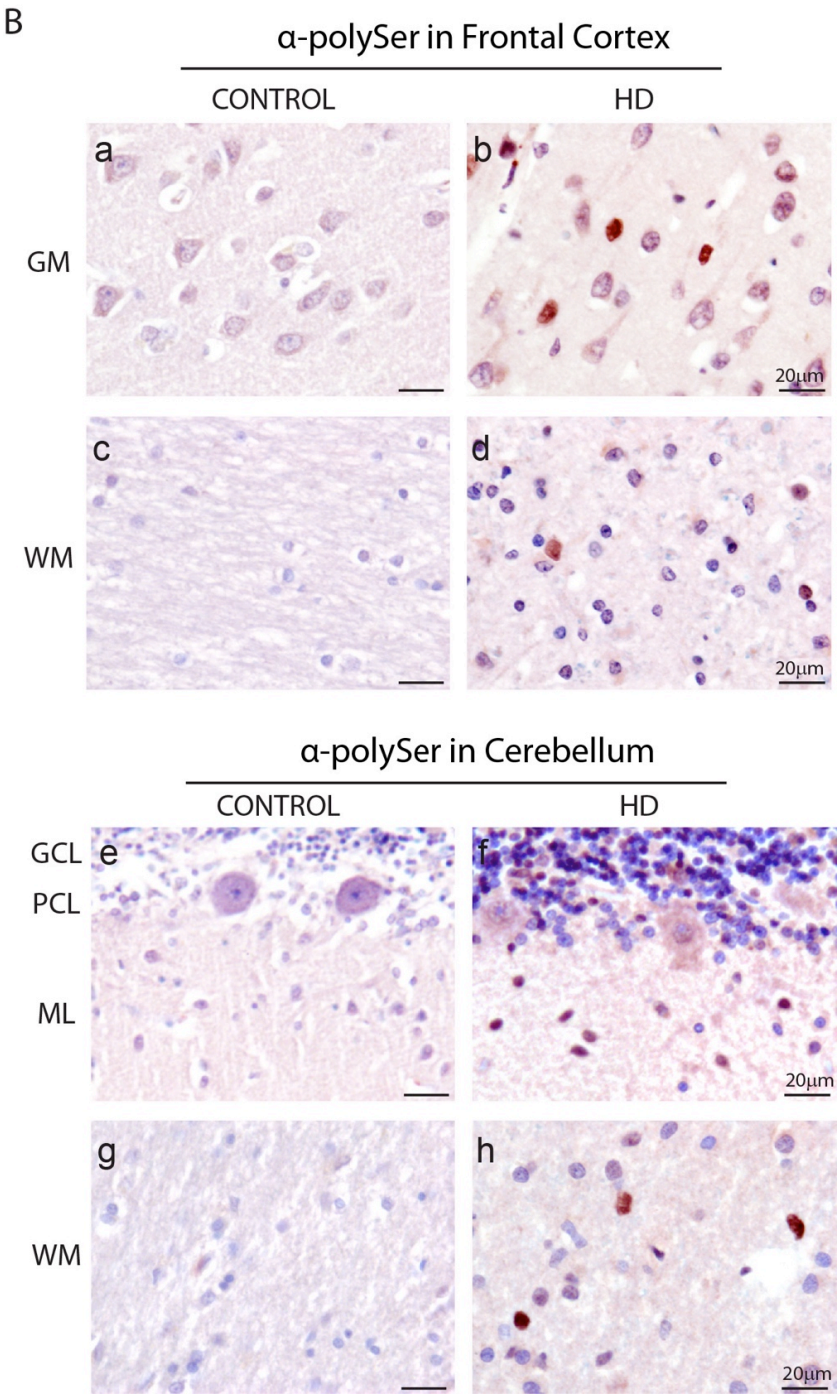


Figure S3B. PolySer RAN proteins in HD frontal cortex and cerebellum. Grey and white matter regions of the frontal cortex show positive α-polySer staining (b,d) in HD but not control autopsy tissue. Cerebellar granular layer, molecular layer and white matter regions also show positive staining for α-polySer in HD (f,h) but not control samples. GM=grey matter, WM=white matter, GCL=granule-cell layer,PCL= Purkinje-cell layer, ML=molecular layer. Red=positive staining, blue=nuclear counterstain.

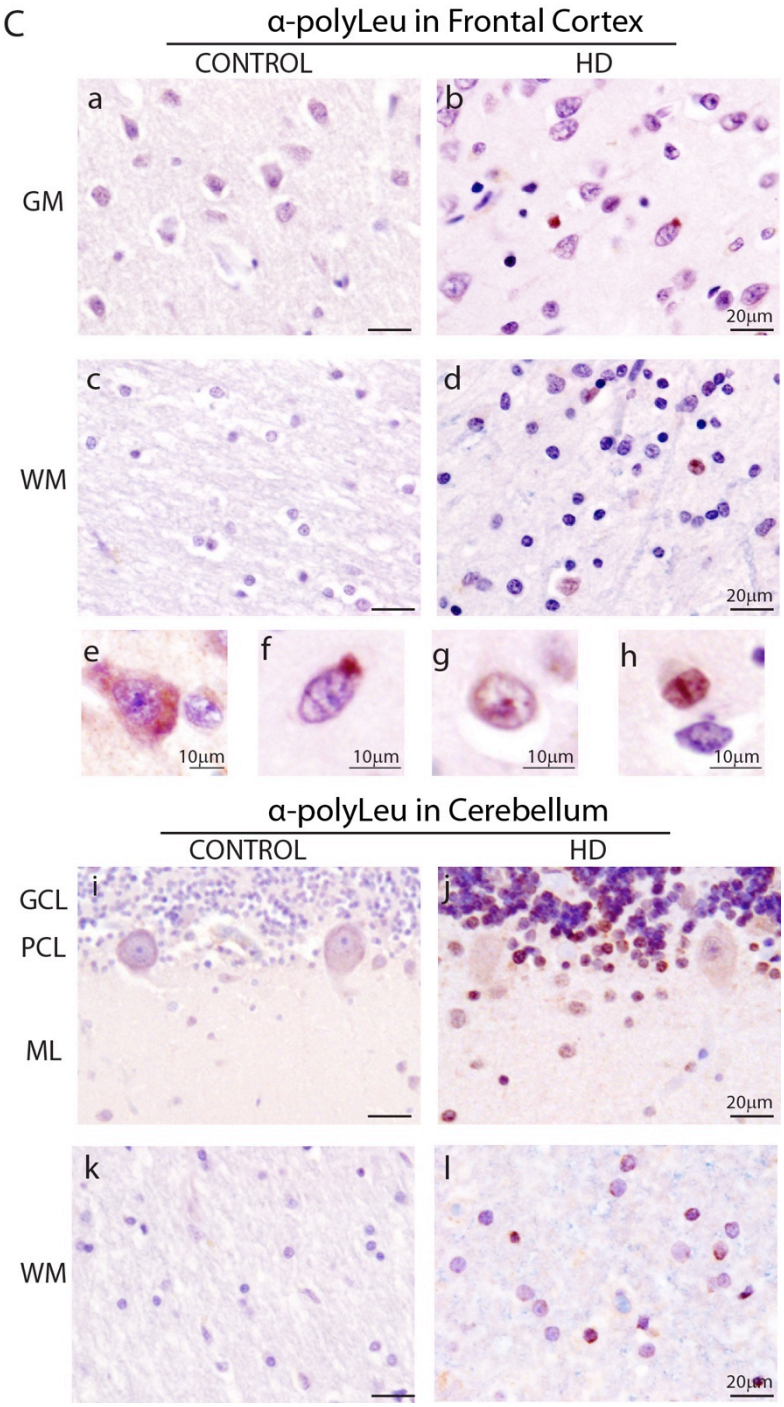


Figure S3C. PolyLeu RAN proteins in HD frontal cortex and cerebellum. Grey and white matter regions of HD but not control frontal cortex show positive nuclear and cytoplasmic IHC staining with α-polyLeu in HD (b,d,e-h). In the cerebellum, α-Leu-Ct staining is found in the granular and molecular layers (j) and white matter (l) in HD but not control (i,k) samples. Diffuse and punctate staining patterns are detected in both the cytoplasm and the nucleus in the frontal cortex (e-h). GM=grey-matter, WM=white matter, GCL=granule-cell layer, PCL= Purkinje-cell layer, ML=molecular layer. Red=positive staining, blue=nuclear counterstain.

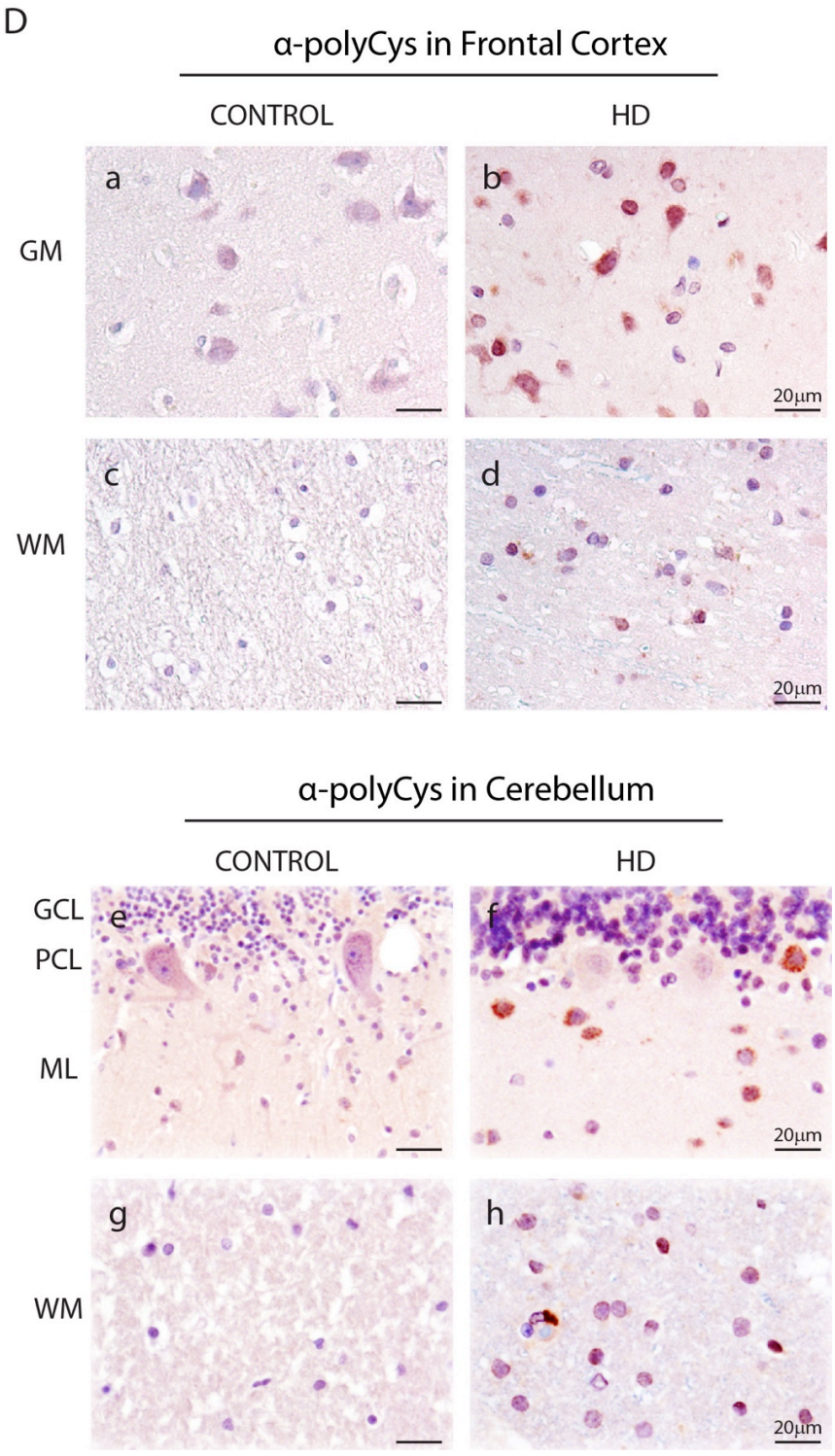


Figure S3D. PolyCys RAN proteins in HD frontal cortex and cerebellum. (a-d) IHC staining shows polyCys positive cells in grey and white matter of HD but not control frontal cortex. (e-h) PolyCys staining shows nuclear accumulation in the cerebellar granular layer and cytoplasmic aggregation in the molecular layer of the cerebellum in HD samples. GM=grey matter, WM=white matter, GCL=granule-cell layer, PCL= Purkinje-cell layer, ML=molecular layer. Red=positive staining, blue=nuclear counterstain.

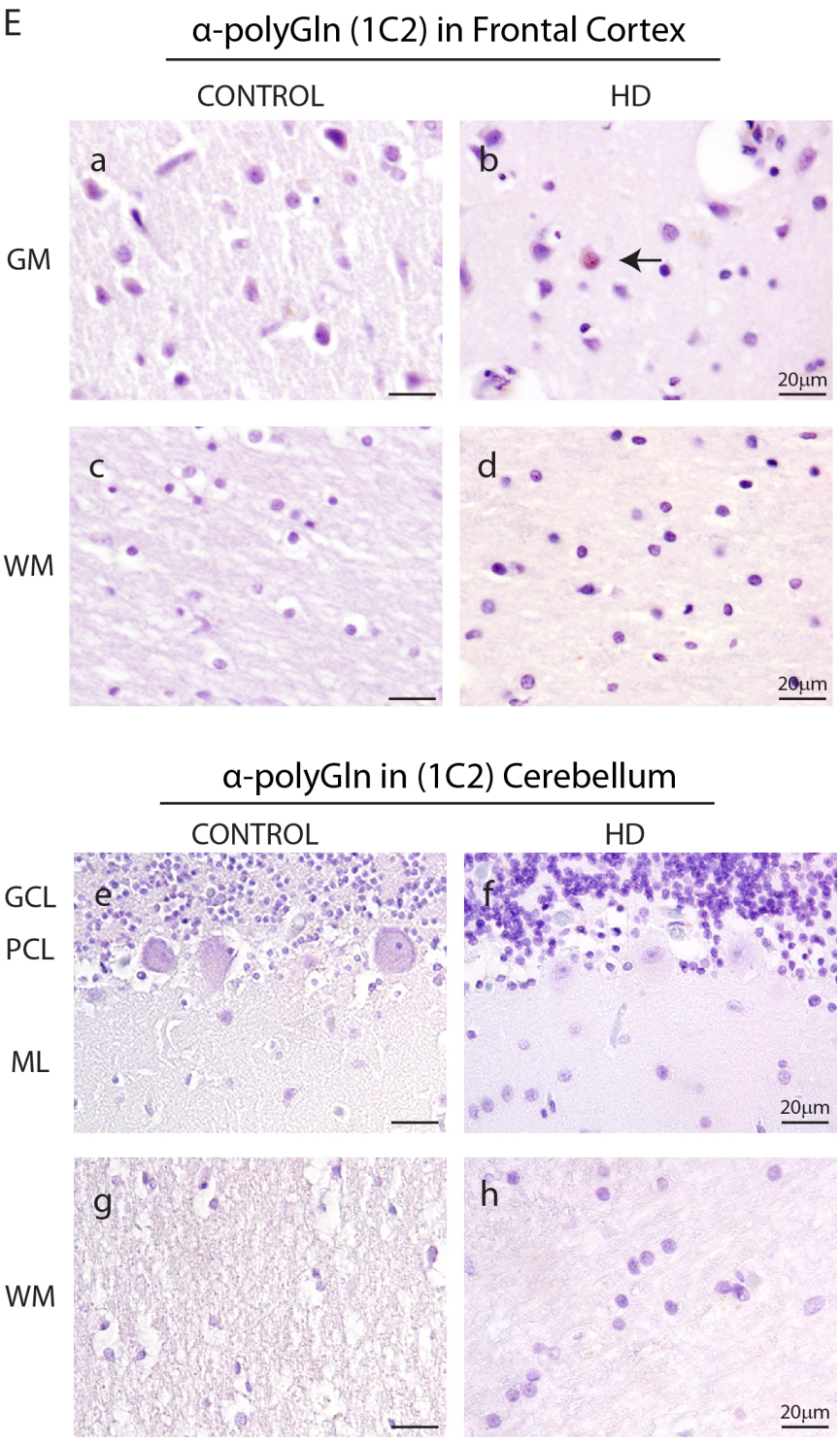
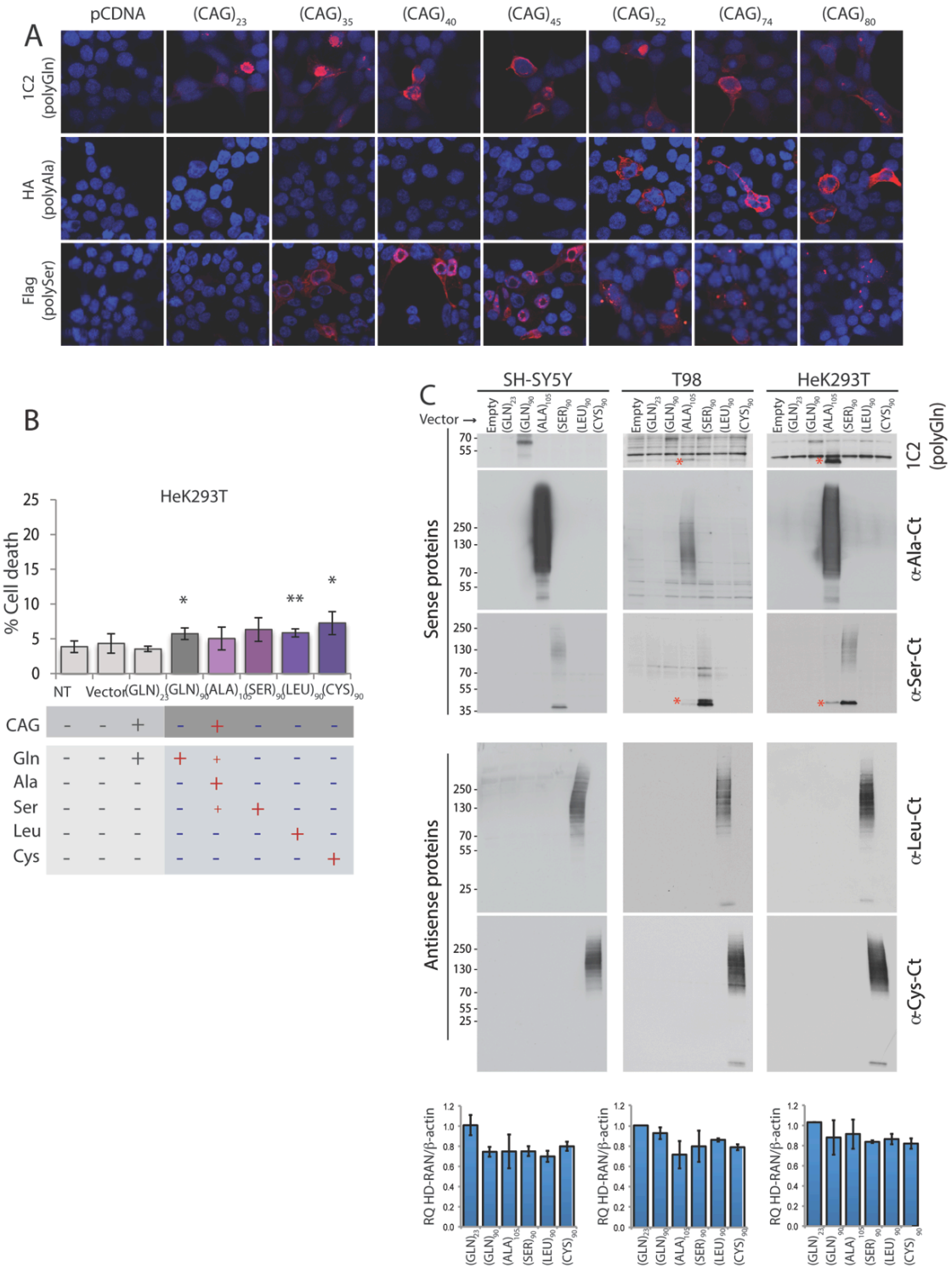


Figure S3E. PolyGln positive staining in HD frontal cortex. (a) IHC staining shows polyGln (1C2) positive neurons in grey matter of HD frontal cortex (b). Subcortical white matter was negative for polyGln in the cases analyzed. Cerebellar tissue didn't show polyGln aggregates at the experimental conditions tested. GM=grey matter, WM=white matter, GCL=granule-cell layer, PCL= Purkinje-cell layer, ML=molecular layer. Red=positive staining, blue=nuclear counterstain.

Supplementary Figure 4.



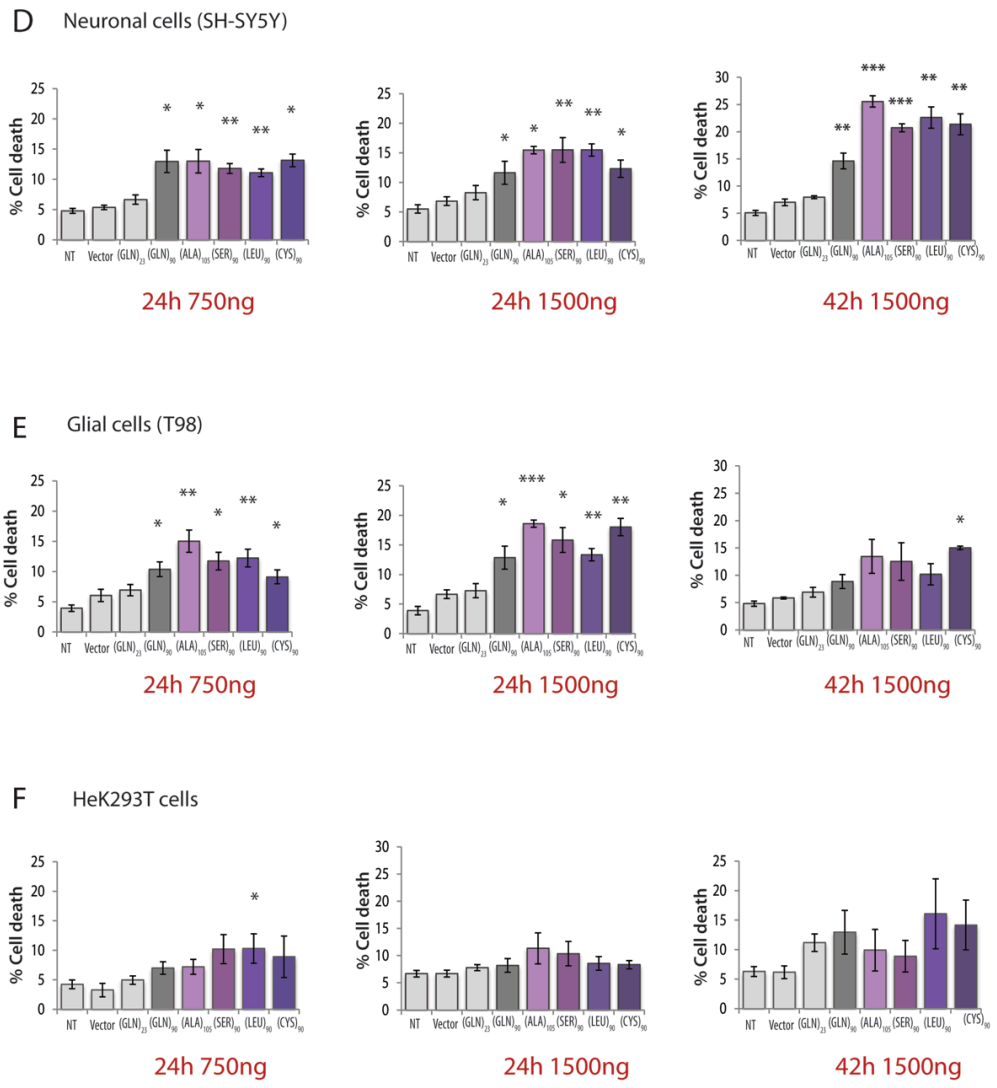


Figure S4. Length-dependent RAN protein accumulation and toxicity. (A) IF (HEK293T cells) showing length dependent accumulation of CAG-encoded HD-RAN proteins in cells transfected with HTT-exon1 constructs. CAG-expanded constructs express polyGln, polyAla and polySer proteins +/- ATG initiation codon. ATG initiated CAA-expanded HTTexon1 constructs only express polyGln protein. (B). Percent cell death of HEK293T cells tranfected with ATG initiated non-hairpin forming codon-substitution minigenes for HD polyGln, polySer, polyLeu, polyCys and ATG initiated GCA-encoded polyAla. Bar graph shows relative cell toxicity 42 hours after transfection evaluated by LDH assay ± SEM (n=5).C. (Top panels) Immunoblots showing protein expression in SH-SY5Y, T98 and HeK293T cells transfected with indicated constructs. PolySer, polyLeu and polyCys codon substitution constructs express single proteins while the ATG(+)-polyAla GCA construct shows robust polyAla and lower leves of RAN polyGln and RAN polySer in HEK293T and T98 (indicated with *). (Bottom panels) qRT-PCR showing transcript levels are comparable with no significant difference between constructs (D-F) Percent death of SH-SY5Y (D), T98 (E) and HEK293T (F) cells after transfection w/ constructs expressing individual HD proteins at indicated vector doses and times post transfection using LDH assays (n=5).

Supplementary Figure 5. HD-RAN and polyGln staining in adult and juvenile-onset cerebellum

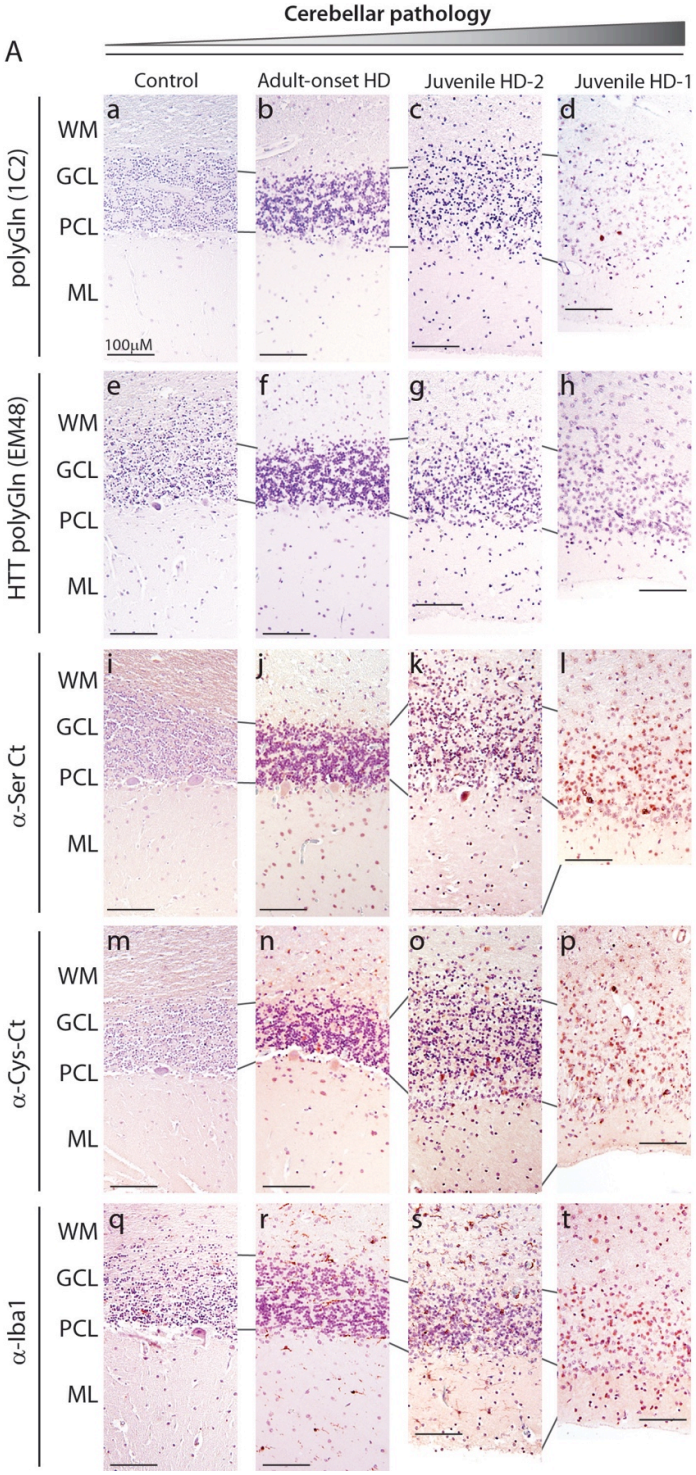


Figure S5 A. IHC staining showing α-polyGln (a-h), α-polySer (i-l), α-polyCys (m-p) and α-Iba-1 (q-t) staining in control, adult-onset and juvenile-onset HD with severe cerebellar atrophy in cerebellar cortex (GCL, PCL, ML) and subcortical white matter (WM). Red=positive staining, blue=nuclear counterstain.

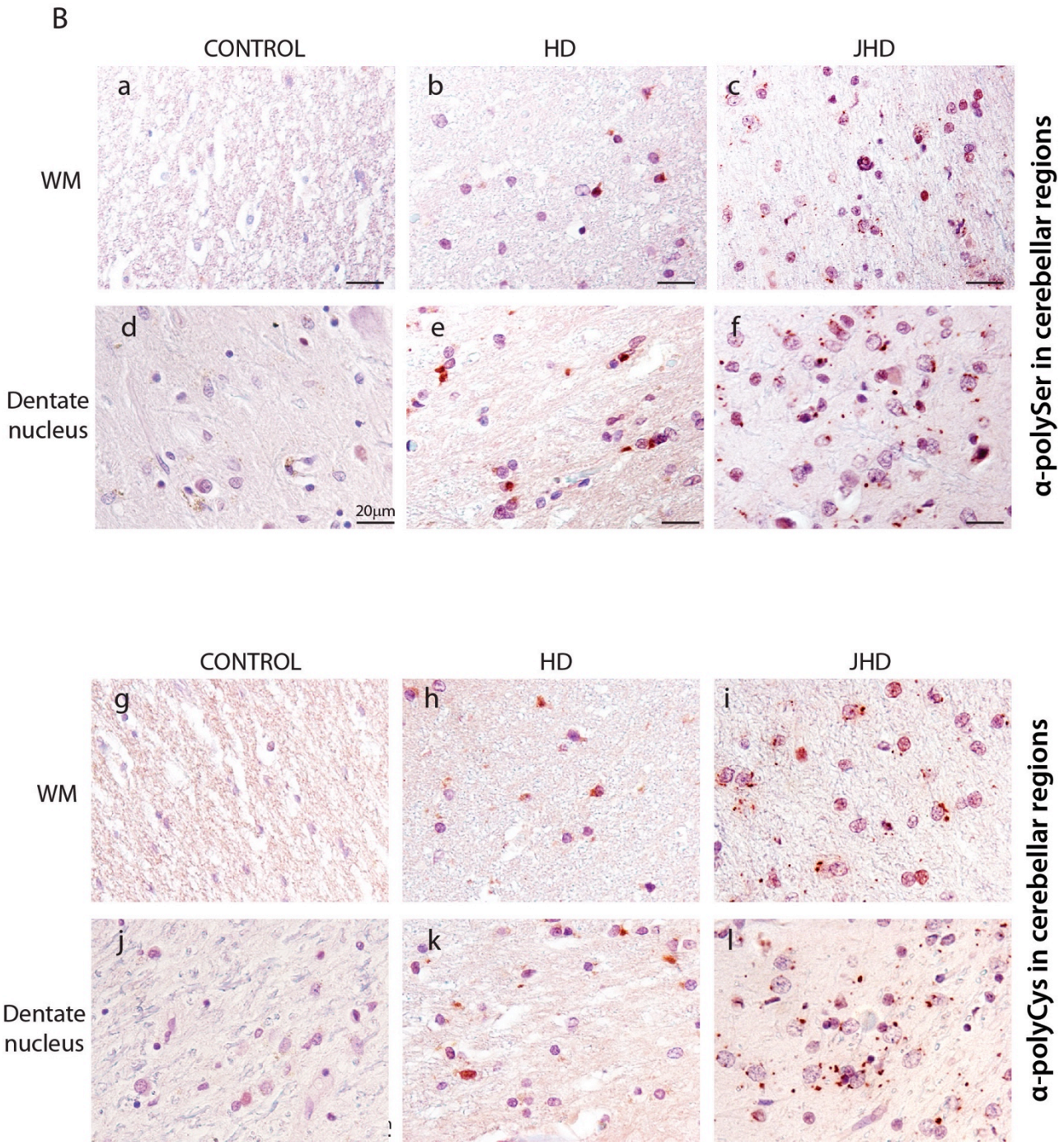


Figure S5. (B) IHC staining in adult- and juvenile-onset HD cerebellar subcortical white matter (a-c, g-i) or the dentate nucleus (d-f, j-l)) with α-polySer-Ct or α-polyCys-Ct antibodies as indicated. Red=positive staining, blue=nuclear counterstain.

Experimental Procedures

cDNA Constructs

HTTexon1 triple tag vectors.

HTTexon1–IRES-GFP expressing vectors differing in CAG repeat length (CAG_{23},CAG_{35}, CAG_{45}, CAG_{52}, CAG_{80}, CAA_{80}) were kindly provided by Drs. Eulalia Marti and Xavier Estivill. HTTexon1 variants were excised using BamHI and EcoRI and subcloned into a modified pcDNA 3.1 vector (Life Technologies) containing a 6xStop codon cassette before the cloning site and three triple epitope tags (Flag, HA, myc) at the C-terminal region.

HTT C-terminal vectors

p3XFlag-HTT-Ct vectors were generated by inserting a triple Flag tag on the pCDNA3.1 vector backbone. EcoRI-$CAG_{23/105}$-BglII minigenes were subsequently inserted right after the 3xFlag region. Additionally, DNA sequence located after the CAG repeat in the human HTT gene were amplified by PCR from human genomic DNA using the primers: HTT-Cterm-BglII-Fw1 (5'-GGAAGATCTAACCTCCTCAGCTTCCTCAGC-3') and HTT-Cterm-BamHI-Rev (5'-CGCGGATCCTGCTGGGTCACTCTGTCTCT-3'). Primers contained overhangs including BglII and BamHI restriction sites. PCR products were inserted into p3xFLAG-CAG digested with the same restriction enzymes to generate constructs expressing ATG-initiated polyGln (CAG) containing the endogenous HTT C-terminal regions (Ct). To generate ATG-initiated polySer-Ct (AGC) or ATG-initiated polyAla-Ct (GCA) constructs, polyGln-Ct vectors were linearized between 3XFlag and CAG expansion regions using XhoI and treated with T4-PNK or mungbean to change the reading frame.

The same approach was followed to generate HTT_{AS} C-terminal vectors. EcoRI- $CTG_{23/105}$-SalI minigenes were inserted into p3XFLAG vectors. HTT_{AS} C-terminal region was cloned using the primers HTT_{AS}-Cterm-SalI-Fw1 (5'-acgcgtcgacgTGGAAGGACTTGAGGGACTC-3') and HTT_{AS}-Cterm-BamHI-Rev1 (5'-cgcggatccCCGCTCAGGTTCTGCTTTTA-3') and inserted into p3XFLAG-CTG vectors to generate ATG-initiated polyLeu (CTG) C-terminal constructs. XhoI digestion and T4-PNK/mungbean treatment was performed to generate ATG-initiated polyCys-Ct (TGC) and ATG-initiated polyAla-Ct (GCT) expressing contructs.

HD-RAN alternative codon vectors

Minigenes for HD polyGln and RAN products were designed, synthesized by ADT Technologies, and inserted into p3XFLAG vectors. The CAG/CTG expansion was substituted by CAA repeats for polyGln, TCTTCC for polySer, CTTCTC for polyLeu and TGT for polyCys to avoid RNA hairpins and prevent RAN translation. Codon substitution was not available for ATG-initiated polyAla constructs, which were generated using AGC repeats.

The integrity of all constructs was confirmed by sequencing.

Production of Polyclonal Antibodies.

The polyclonal antibodies were generated by New England Peptide. The α-$HD_{CAG\text{-}ALA\ \&\ SER}$ antisera were raised against synthetic peptide corresponding to the C-Termini of the predicted polyAla and polySer frames of HD in the CAG direction: APAAAPAATRPG and RPRRHPARLWLRSR respectively. The α-$HD_{CTG\text{-}CYS\ \&\ LEU}$ were raised against synthetic peptide corresponding to the C-termini of the predicted polyCys and polyLeu frames of HD in the CTG direction: KDLRDSKAFISFSR and GLGPTRGAAQHRG respectively.

Cell Culture and Transfection

SH-SY5Y (human neuroblastoma), T98 (human glioblastoma) and HEK293T (human embryonic kidney cells) were maintained under standard conditions of temperature (37°C), humidity (95%), and carbon dioxide (5%) and grown in Dulbecco's Modified Eagle's Medium (DMEM, Life technologies) supplemented with 10% FBS (Fetal Bovine Serum, Corning cellgro), 100 units/ml penicillin and 100 µg/ml Streptomycin (Corning cellgro).Transfection experiments were conducted using Lipofectamine 2000 (Life technologies), according to the manufacturer's instruction and at a 60% cell confluence. Cells were plated 24 hours before transfection.

Human Autopsy Tissue

Control and HD autopsy tissue was collected at Johns Hopkins and the University of Florida with informed consent of patients or their relatives and approval of local institutional review boards.

Immunofluorescence

T98 and HEK293T cells were grown on coverslides. At the indicated time after transfection, cells were rinsed with PBS and fixed for 30 min at room temperature with 4% paraformaldehyde in PBS. Cells were then washed in PBS and permeabilized for 30 min in 0.5% Triton-X-100 in PBS. Non-specific binding was blocked by incubation in 10%FBS in PBS for 1 hour. Incubation with the indicated primary antibody dilutions was carried out overnight at 4°C in PBS containing 1% FBS. After washing three times in PBS 1X, coverslides were incubated with secondary anti-mouse IgG Alexa 488 or IgG Alexa 594 (Molecular Probes) at a dilution of 1:2000 for 1 hour at room temperature. Coverslides were washed and mounted in Vectashield-DAPI (Invitrogen), and cells visualized under a Leica confocal microscope. Images were captured using a digital camera (Leica TCS SP5). Primary antibodies used were anti-polyQ (MAB1574, 1:2000, Millipore), anti-Flag M2 (1:1500, Sigma), anti-HA (1:1500, Covance). α-Ala-Ct, α-Ser-Ct, α-Leu Ct and α-Cys Ct were used at 1:1000, 1:500, 1:1500 and 1:500 respectively.

Immunohistochemistry

For the detection of HD-RAN proteins using C-terminal antibodies the following protocol was followed under harsh antigen exposure conditions. Eight-micrometer sections were deparaffinized in xylenes and rehydrated through an alcohol gradient. Subsequent antigen retrieval steps were performed. First, 1ug/mL proteinase K treatment in 1mM $CaCl_2$, 50mM Tris buffer (pH=7.6) for 40 minutes at 37°C. Second, pressure cooked in 10mM EDTA (pH=6.5) for 15minutes. Third, 95%

formic acid treatment for five minutes(Christensen et al., 2009; Kai et al., 2012). Endogenous peroxidase block was performed in 3%H_2O_2 methanol for ten minutes. To block nonspecific binding a nonserum block (Biocare Medical) was applied for 15 minutes. Primary antisera were applied in 1:10 non-serum block at 4C overnight; α-polyAla (1:5000), α-polySer (1:4500), α-polyCys (1:2500), and α-polyLeu (1:6000). Rabbit Linking Reagent (Covance) was applied for 30 minutes at room temperature. Secondary antibodies were Biotin-Avidin/Streptavidin labeled using ABC reagent (Vector laboratories, Inc.) and detection performed by exposure to Vector Red Substrate Kit (Vector Laboratories, Inc.). Slides were finally dehydrated and mounted using Cytoseal 60 (Electron microscopy sciences).

Double staining experiments were carried out using the same protocol detected by DAB peroxidase substrate kit (Vector labs) for all the RAN antibodies. The second round used Iba-1 or Caspase 3 antibodies, that were labeled by 4+ Streptavidin AP label (Biocare medical) and detected using Vector Blue substrate kit (Vector labs) for alkaline phosphatase. Counterstain was performed using Nuclear Fast Red solution (Sigma) for 20 minutes at room temperature. Slides were dehydrated using ethanol and Histoclear (National Diagnostics) and mounted using VectaMount mounting medium (Vector labs). All the washing steps were done in 100mM This-HCl pH 8.2. Additional primary antibodies used were: α-Iba1 (Abcam, goat, 1:1000), α-active Caspase-3 (Abcam, rabbit, 1:300), EM48 (Millipore, mouse, 1:75), 1C2 (Millipore, mouse, 1:10000 for staining in striatum and cortex; 1:10000/1:3000/1:1000 for cerebellar staining).

IHC staining is summarized in Table 1 with representative examples from the following cases shown in the following figures: Figure 1D-e (HD-4); Figure 1Df (HD-4); Figure 1D-g (HD-7); Figure 1D-h (HD-7); Figures 2C-E (HD-4); Figures 2F-I (HD-7); Figure 3 polyGln, Ala and Cys (HD-4), Figure 3 polySer (HD-7); Figure 3 polyLeu (HD6); Figure 5 (HD4 and JHD1); Figures S2A,B,E,F (HD-4); Figures S2C,D,G (HD-7); Figures S3A,D,E (HD-4); Figure S5A (HD4, JHD1, JHD2); Figure S5B (HD7, JHD1).

Immunoblotting.

Cells in each well of a six-well tissue-culture plate were rinsed with PBS and lysed in 90 μL RIPA buffer for 3 minutes on ice. The cell lysates were collected and centrifuged at 16,000 x g for 15min at 4°C. The protein concentration of the supernatant was determined using the protein assay dye reagent (Bio-Rad). Twenty micrograms of protein were separated in a 4-12% NuPage Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane (Amersham). The membrane was blocked in PBS containing 0.05% Tween-20 detergent (PBS-T) and 5% dry milk powder. Primary antibodies were prepared in 1%milk in PBS-T and incubated overnight at 4°C. After washing with PBS-T, membranes were incubated with secondary antibodies (1:3000 in PBS-T) for 1 hour at room temperature and washed with PBS-T. Detection was performed using Western Lightning Plus-ECL (Perkin Elmer).

Primary antibodies were anti-polyQ (MAB1574, 1:2000, Millipore), anti-Flag M2 (1:3000, Sigma), anti-HA (1:2000, Covance). α-Ala-Ct, α-Ser-Ct, α-Leu Ct and α-Cys Ct were used at 1:2000, 1:750, 1:1500 and 1:750 respectively. Anti-GAPDH (1:5000, ab8245 Abcam) was used as loading control.

Secondary antibodies were peroxidase-conjugated anti-mouse and anti-rabbit (1:3000, GE Healthcare). Membranes were blocked in 3% BSA PBS-T for α-polyCys-Ct incubations.

Western blots shown in Figure 1 are from cases HD-4, HD-7.

In general RAN proteins migrate higher than their predicted MW on SDS PAGE gels and the inclusion of non-repeat sequence, even if it would be expected to increase the MW of the protein can alter its migration pattern. For example, the migration pattern of the +ATG HTT polyQ shown in Figure 1B, which starts at the ATG initation codon is predicted to contain 17 additional N-terminal amino acids compared to a polyGln RAN initiated protein expected to begin at or close to the 5' end of the CAG expansion. Based on similar studies done in SCA8 (Zu et al., 2011), we believe that the inclusion of non-repetitive sequences in these homopolymeric expansion proteins typically increases the solubility of the protein making it migrate further into the gel.

Cell-Toxicity Assays

Cell toxicity was determined measuring Lactate dehydrogenase release from dying cells (Cytotox 96, Promega) following manufacturer's protocol. LDH determinations were performed in five independent experiments each performed in quintuplicates and measured at 490nm.

RNA quantification for cell-toxicity assays

Total RNA from transfected cells was isolated using miRNeasy Mini kit (Qiagen) following the manufacturer's instructions. RNA was retrotranscribed using the SuperScript III RT kit (Invitrogen) and random-hexamer primers. HD-RAN alternative codon cDNAs were amplified using iQ™ SYBR® Green Supermix (Biorad) using the primers: 3xFLAG Forward; 5′-ACCTCCTCAGCTTCCTCAGC-3′, sFLAG Reverse: (5′-GCTGGGTCACTCTGTCTCTG-3′). β-Actin was used as a reference gene and was amplified using the primers: ACTB-3: 5′-CTGGAACGGTGAAGGTGACA-3′; ACTB-4: 5′-GGGAGAGGACTGGGCCATT-3′.q-RT-PCR results were analyzed using the 2−delta delta Ct method.

Statistical analysis

Statistical significance was calculated using the two-tailed unpaired t-student's test for single comparisons ($p<0.05$) and the analysis of variance (ANOVA) for the comparison of multiple pair-wise conditions. "n" refers to independent experiments.

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